Journal of Cell Science

Macrophages show higher levels of engulfment after disruption of *cis* interactions between CD47 and the checkpoint receptor SIRPα

Brandon H. Hayes, Richard K. Tsai, Lawrence J. Dooling, Siddhant Kadu, Justine Y. Lee, Diego Pantano, Pia L. Rodriguez, Shyamsundar Subramanian, Jae-Won Shin and Dennis E. Discher
DOI: 10.1242/jcs.237800

Editor: Ana-Maria Lennon-Duménil

Review timeline
Original submission: 14 August 2019
Editorial decision: 25 September 2019
First revision received: 20 November 2019
Accepted: 20 December 2019

Original submission

First decision letter

MS ID#: JOCES/2019/237800

MS TITLE: Macrophages eat more after disruption of *cis* interactions between CD47 and the checkpoint receptor SIRPα

AUTHORS: Dennis E. Discher, Brandon Hayes, Richard Tsai, Diego Pantano, Jae-Won Shin, Pia Rodriguez, and Shyamsundar Subramanian
ARTICLE TYPE: Short Report

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers gave favourable reports but raised some critical points that will require amendments to your manuscript. I hope that you will be able to carry these out, because I would like to be able to accept your paper.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field
The manuscript by Hayes et al. is a study of the interaction between CD47 and SIRPa on macrophages. Treatments that block the interaction between CD47 and SIRPa suppress tumor

growth via different mechanisms including enhanced phagocytosis. In that case, the inhibitory receptor SIRPa does not engage with CD47 and is less active to prevent phagocytosis of the CD47-positive target cell. The role of the CD47 molecules expressed on macrophages has been overlooked so far and this is the focus of the present study.
The authors convincingly demonstrated that CD47 inhibits phagocytosis via SIRPa in cis in model macrophage-like cells. The experiments are well controlled. The manuscript is of very good scientific standards and is well written. The results will be of interest in the field of immunomodulation but also form a cell biology point of view on receptor-receptor interactions.

Comments for the author

- The authors have used the THP1 monocytic cell line with PMA differentiation for 2 days. This is not sufficient to call the cells “macrophages”. A longer PMA treatment followed by a resting culture is necessary to obtain macrophage-like cells, as reported in [Daigneault M, et al. (2010) The Identification of Markers of Macrophage Differentiation in PMA-Stimulated THP-1 Cells and Monocyte-Derived Macrophages. PLoS ONE 5(1): e8668. doi:10.1371/journal.pone.0008668.]
The authors could use this protocol, and at least change the text to talk about "macrophage-like cells".

- The phagocytic index reported for THP1 cells is low (1-2) in figure 2. The receptor densities and interactions might be different in primary macrophages that usually exhibit a higher rate (number of targets and speed) of phagocytosis. Since the authors have access to blood and performed work in PBMCs, they could use primary human monocyte-derived macrophages. These cells can easily be treated with siRNA and should be used to confirm the experiments presented in Figure 2.

Reviewer 2

Advance summary and potential significance to field

Hayes et al. report the interesting finding that SIRPα-CD47 cis interactions on the surface of a macrophage, rather than just trans interactions with target cells, play an important role inhibiting macrophage phagocytosis. They show that preventing these interactions—either through blocking CD47 with therapeutic blocking antibodies or by knocking down CD47 expression—prevents cis inhibition and boosts macrophage phagocytosis of both opsonized RBCs and opsonized beads. Reduction of SIRPα-CD47 cis interactions is shown to reduce SIRPα phosphorylation and thereby prevents subsequent inhibitory signaling. The authors also report an increase in apparent SIRPα affinity for soluble CD47 in the absence of cis interactions and use simulations to argue that SIRPα-CD47 cis binding is possible. These findings are both interesting and potentially important, but further data and control experiments are needed to support the significance of inhibitory CD47-SIRPα cis interactions.

Comments for the author

In particular, the following issues should be addressed:

1. In Figure 1D, blocking CD47 on only the macrophage (not the RBC) should release SIRPα molecules to interact in trans, presumably increasing inhibitory interactions with CD47 on the RBC, yet eating goes up. What would account for this increase?

2. Phagocytosis assays in Figures 1 and 2 should include additional conditions and controls to further support the effects of cis interactions. In Figure 1D it would be helpful to show what happens when (i) the macrophage AND RBC are pre-treated with CD47 antibody in the absence of RBC opsonization and (ii) only the macrophage is pre-treated with CD47 blocking antibody in the presence of RBC opsonization. In Figure 2D, phagocytosis of both shRBCs and microbeads should be performed in the absence of opsonization to validate that CD47 knockdown has not changed background phagocytosis.

3. Two different metrics of phagocytosis are used, and it is not clear why. In Figures 1C, D "% Macrophage eating" is the metric, while in Figure 2 "# shRBCs per Macrophage" is the metric. It seems that the data in both figures could be reported with either metric. Is there a reason for the use of different metrics?

4. In Figure 3, why does the anti-CD47 antibody not compete off SIRPα bound in trans? It seems that this competition should be the same as when pre-priming the macrophage with anti-CD47 antibody, in which case you see strong CD47 antibody binding. Reporting the anti-CD47 antibody binding affinity would also be helpful.

5. It would be helpful to report the absolute densities of CD47 and SIRPα molecules on the different cell types in Figure 1B. Also, labeling SIRPα and quantifying surface expression would be useful in validating the claims of this experiment in Figure 3A, and it could also validate that the CD47-SIRPα stoichiometry is in a reasonable and physiological range on the CHO cells.

6. The authors use simulations to show that SIRPα-CD47 cis binding is possible in Figure 4 and S4B, but the likelihood of binding is cis vs trans is unclear. The authors' simulations would benefit from analysis of the energetic cost of bending versus binding, and an evaluation of how likely it is to find SIRPα molecules in this state. It seems at least possible that SIRPα on the macrophage would prefer to bind to CD47 on the RBC surface rather than CD47 on the macrophage if both were nearby (i.e. trans interactions could outcompete cis interactions).

7. How does CD47 binding to SIRPα in cis drive phosphorylation of the ITIM domain?

8. Minor points:
a. It appears that the labels are switched for the two conditions in Figure 3B and C. Additionally, it is unclear how the data points for these plots were selected from flow cytometry data. Are they averages of populations?

b. Figure S3C – The y-axis is incorrect.

First revision

Author response to reviewers' comments

Editor's Comments and Responses:
We have now reached a decision on the above manuscript. As you will see, the reviewers gave favourable reports

RESPONSE:
We sincerely thank the Editor and both Reviewers for the careful read and the comments as well as the time spent on our Short Report.

Editor: …but raised some critical points that will require amendments to your manuscript. I hope that you will be able to carry these out, because I would like to be able to accept your paper. Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

RESPONSE:
TEXT CHANGES are in red font or so indicated within the 'Response to Reviewers' box, which also includes a point-by-point response detailing how we have dealt with the points raised.

New experimental results and analyses have been added as:

Fig. 1B-table:
Quantifies receptor densities as requested by Reviewer-2.

Figs. 3A-D, S4:
Uses primary macrophages in response to comments from Reviewer-1.

Fig. S1B:
Documents similarity of THP-1 macrophages to primary macrophages in response to Reviewer-1.

Fig. S2C:
Additional phagocytosis conditions show expected trends – as requested by Reviewer-2.

Fig. S3C-D:
Background phagocytosis shown for THP-1 WT & KD cells as requested by Reviewer-2.

RESPONSE:
We hope that the revision is found to be responsive.
Reviewer 1 Comments and Responses:

Advance summary and potential significance to field
The manuscript by Hayes et al. is a study of the interaction between CD47 and SIRPa on macrophages. Treatments that block the interaction between CD47 and SIRPa suppress tumor growth via different mechanisms including enhanced phagocytosis. In that case, the inhibitory receptor SIRPa does not engage with CD47 and is less active to prevent phagocytosis of the CD47-positive target cell. The role of the CD47 molecules expressed on macrophages has been overlooked so far and this is the focus of the present study.
The authors convincingly demonstrated that CD47 inhibits phagocytosis via SIRPa in cis in model macrophage-like cells. The experiments are well controlled. The manuscript is of very good scientific standards and is well written. The results will be of interest in the field of immunomodulation but also form a cell biology point of view on receptor-receptor interactions.

RESPONSE:
We sincerely thank Reviewer-1 for the careful read and the comments as well as the time spent on our Short Report.

Newly added Figures 3 and S4 address some of the Reviewer's specific comments below but also reflect the Reviewer's sentiment in the last comment that: "The results will be of interest in the field of immunomodulation but also form a cell biology point of view on receptor-receptor interactions." As the reviewer will see below in the abstract and the Results section:

TEXT CHANGES:
Abstract
… Many cell types express both SIRPα and CD47, including mouse melanoma B16 cells, and CRISPR-mediated deletions modulate B16 phagocytosis consistent with cis-trans competition. …

Results and Discussion
"SIRPα on cancer cells modulates their engulfment
Numerous cell types express both CD47 and SIRPα, including human A549 lung cancer cells (Fig.1B) and mouse B16 melanoma cells (Fig.S1B, S4A). We hypothesized that with such cells cis CD47-SIRPα interactions regulate their phagocytosis by modulating trans presentation of 'self' (Fig.3A) – as is relevant to cancer therapy (Fig.1A). B16 were studied to generalize species effects and because B16s are widely used in preclinical immunotherapy, including CD47-SIRPα blockade (Ingram et al., 2017; Chowdhury et al., 2019; Mandal et al., 2019).
CRISPR/Cas9-mediated knockout (KO) of CD47 or SIRPα in B16 cells used a non-targeting guide-RNA for control, and all B16 cells were equally opsonized with anti-Tyrp1 (Fig.S1B,S4A) before adding to mouse bone-marrow derived macrophages (BMDMϕs). Flow cytometry and imaging both show SIRPα-KO's were engulfed less than controls (by ~0.3-0.4-fold), compared to the 2-3-fold increase for

CD47-KO's (Fig.3B-D;-S4B); also 1-2 cells were again engulfed per BMDMϕ (Fig.3C,D). These results support the general hypothesis that CD47-SIRPα cis interactions modulate trans interactions and address a conceivable mechanism in the phagocytosis results (Fig.1D,2A,S2B,C) in that blocking CD47 on only the macrophage should release SIRPα molecules to interact in trans and increase inhibition – yet eating goes up. While consistent with suppression of baseline inhibition (Fig.2B), further study is needed."

Reviewer 1: The authors have used the THP1 monocytic cell line with PMA differentiation for 2 days. This is not sufficient to call the cells "macrophages". A longer PMA treatment followed by a resting culture is necessary to obtain macrophage-like cells, as reported in [Daigneault M, et al. (2010) The Identification of Markers of Macrophage Differentiation in PMA-Stimulated THP-1 Cells and Monocyte-Derived Macrophages. PLoS ONE 5(1): e8668. doi:10.1371/journal.pone.0008668.] The authors could use this protocol, and at least change the text to talk about macrophage-like cells.

RESPONSE:
We have edited the text and figures to more clearly indicate 'THP-1 macrophages', except where we study primary macrophages in newly added Figures 3, S1B, S4. The Methods are also expanded to reference several very recent papers (including one in BMC Cancer 2015, one in Cell Host & Microbe 2018, and another in PLoS One 2018) relative to the cited PLoS One 2010 paper, and the recent papers use the 'macrophage' nomenclature consistent with newly added expression data (in Fig.S1B).

TEXT CHANGE:
Materials and Methods
Cell cultures and THP-1 differentiation

Differentiation of THP-1 cells followed a protocol based on several recent publications that all refer to the differentiated cells as THP-1 macrophages (Genin et al., 2015; Starr et al., 2018; Sedlyarov et al., 2018). Differentiation was achieved in 100 ng/mL phorbol myristate acetate (PMA) (Millipore Sigma) for 2-3 days, which leads to these cells switching from growth in suspension (as monocytic cells) to attaching to tissue-culture plastic. Fig.S1B's gene expression data confirms PMA-differentiated THP-1 macrophages and primary macrophages have similar expression profiles for key macrophage markers, while differing greatly from two epithelial cell types.

Primary mouse bone marrow-derived macrophages (BMDMϕs) and phagocytosis of B16 cell lines
Bone marrow cells were isolated from femurs and tibias of healthy male C57BL6/J mice (The Jackson Laboratory 000664) and cultured in 10-cm petri dishes containing Iscove's Modified Dulbecco's Media (IMDM, Gibco 12440-053) supplemented with 10% v/v fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin (Gibco), and 20 ng/mL M-CSF (Biolegend 576404) for 7 d at 37 °C, 5% CO2. The resulting BMDMϕs were detached with 0.05% trypsin-EDTA (TRED, Gibco) and replated in 6- or 24-well tissue culture plates at a density of 2.1 x 104 cells/cm2.

Reviewer 1: The phagocytic index reported for THP1 cells is low (1-2) in figure 2.

RESPONSE:
We must first clarify that while Figure 2A indeed indicates the number of 'engulfed targets per macrophage', this is not the "phagocytic index" as reported in Figure S2A-bottom, which reflects 'total eating' as the product of 'engulfed targets per macrophage' and '% macrophages that eat'.

Reviewer 1: The receptor densities and interactions might be different in primary macrophages that usually exhibit a higher rate (number of targets and speed) of phagocytosis. Since the authors have access to blood and performed work in PBMCs, they could use primary human monocyte-derived macrophages. These cells can easily be treated with siRNA and should be used to confirm the experiments presented in Figure 2.

RESPONSE:
New Figures 3, S4 use 'primary monocyte-derived macrophages' from mouse in phagocytosis experiments. Unfortunately, use of primary human cells requires a new IRB protocol that would

require many months for approval. We used CRISPR knockout cells as targets of engulfment, because the chosen cancer cells express both CD47 and SIRPa, and consistent with our Figure 2A and S2A results showing 1-2 targets per THP-1 macrophage, for bone-marrow derived macrophage (BMDMϕ):

TEXT CHANGE:
"1-2 cells were again engulfed per BMDMϕ (Fig.3C,D)."

Primary macrophages are difficult to simply 'treat with siRNA', but our new Results for primary mouse macrophage engulfment of CRISPR knockout cancer cells helps generalize findings to different species and different cell types, in keeping with Journal of Cell Science and a broad "cell biology point of view on receptor-receptor interactions":

TEXT CHANGES (as above):
Abstract
… Many cell types express both SIRPα and CD47, including mouse melanoma B16 cells, and CRISPR-mediated deletions modulate B16 phagocytosis consistent with cis-trans competition. …
Results and Discussion
"SIRPα on cancer cells modulates their engulfment
Numerous cell types express both CD47 and SIRPα, including human A549 lung cancer cells (Fig.1B) and mouse B16 melanoma cells (Fig.S1B, S4A). …
… These results support the general hypothesis that CD47-SIRPα cis interactions modulate trans interactions ..."
Reviewer 2 Comments and Responses:

Reviewer 2 - Advance summary and potential significance to field
Hayes et al. report the interesting finding that SIRPα-CD47 cis interactions on the surface of a macrophage, rather than just trans interactions with target cells, play an important role inhibiting macrophage phagocytosis. They show that preventing these interactions—either through blocking CD47 with therapeutic blocking antibodies or by knocking down CD47 expression—prevents cis inhibition and boosts macrophage phagocytosis of both opsonized RBCs and opsonized beads. Reduction of SIRPα-CD47 cis interactions is shown to reduce SIRPα phosphorylation and thereby prevents subsequent inhibitory signaling.

The authors also report an increase in apparent SIRPα affinity for soluble CD47 in the absence of cis interactions and use simulations to argue that SIRPα-CD47 cis binding is possible. These findings are both interesting and potentially important, but further data and control experiments are needed to support the significance of inhibitory CD47-SIRPα cis interactions.

RESPONSE:
We sincerely thank Reviewer-2 for the careful read and the comments as well as the time spent on our Short Report.

New experiments have been added to the Results to address the various concerns. These include a major new experimental approach "to support the significance of inhibitory CD47-SIRPα cis interactions":

TEXT CHANGES:
Abstract
… Many cell types express both SIRPα and CD47, including mouse melanoma B16 cells, and CRISPR-mediated deletions modulate B16 phagocytosis consistent with cis-trans competition. …
Results and Discussion
"SIRPα on cancer cells modulates their engulfment
Numerous cell types beyond phagocytes express both CD47 and SIRPα, including human A549 lung cancer cells (Fig.1B) and mouse B16 melanoma cells (Fig.S1B, S4A), …
… These results support the general hypothesis that CD47-SIRPα cis interactions modulate trans interactions…"

Reviewer 2: In Figure 1D, blocking CD47 on only the macrophage (not the RBC) should release SIRPα molecules to interact in trans, presumably increasing inhibitory interactions with CD47 on the RBC, yet eating goes up. What would account for this increase?

RESPONSE:
Our new experimental results with cancer cells helps address this interesting possibility by an alternative approach, and we acknowledge this at the end of this new section:

TEXT CHANGES:
Results and Discussion
"SIRPα on cancer cells modulates their engulfment
…
CRISPR/Cas9-mediated knockout (KO) of CD47 or SIRPα in B16 cells used a non-targeting guide-RNA for control, and all B16 cells were equally opsonized with anti-Tyrp1 (Fig.S1B,S4A) before adding to mouse bone-marrow derived macrophages (BMDMϕs). Flow cytometry and imaging both show SIRPα-KO's were engulfed less than controls (by ~0.3-0.4-fold), compared to the 2-3-fold increase for CD47-KO's (Fig.3B-D;-S4B); also 1-2 cells were again engulfed per BMDMϕ (Fig.3C,D). These results support the general hypothesis that CD47-SIRPα cis interactions modulate trans interactions and address a conceivable mechanism in the phagocytosis results (Fig.1D,2A,S2B,C) in that blocking CD47 on only the macrophage should release SIRPα molecules to interact in trans and increase inhibition – yet eating goes up. While consistent with suppression of baseline inhibition (Fig.2B), further study is needed."

Reviewer 2: Phagocytosis assays in Figures 1 and 2 should include additional conditions and controls to further support the effects of cis interactions. In Figure 1D, it would be helpful to show what happens when (i) the macrophage AND RBC are pre-treated with CD47 antibody in the absence of RBC opsonization and (ii) only the macrophage is pre-treated with CD47 blocking antibody in the presence of RBC opsonization.

RESPONSE:
New experimental data in Fig.S2C (Experiment B) includes the requested conditions, comparing to Fig.1D to show the expected trend:

TEXT CHANGE:
"pre-binding of anti-CD47 to THP-1 macrophages tends to increase the percent eating by an amount similar to that achieved with anti-CD47 blockade on fully opsonized hRBCs (Fig.1D, S2C)."

Reviewer 2: In Figure 2D, phagocytosis of both shRBCs and microbeads should be performed in the absence of opsonization to validate that CD47 knockdown has not changed background phagocytosis.

RESPONSE#1:
First, Fig.S3C shows wild-type and knockdown control THP-1 cells with no significant difference in eating. Opsonized conditions are needed for the most reliable assessments of significant eating. Secondly, new Fig.S3D-right shows for human-RBC the expected trends for background eating:

TEXT CHANGE:
Compared to the ShRBCs, opsonized human-RBCs show a decrease in 'self'-inhibited background eating by both WT and KD THP-1 macrophages, following the expected trends (Fig. S3D).

RESPONSE#2:
Perturbing macrophages is complex and further motivated the study of B16 knockouts:

TEXT CHANGE:
Numerous cell types express both CD47 and SIRPα, including … mouse B16 melanoma cells (Fig.S1B, S4A). We hypothesized that with such cells cis CD47-SIRPα interactions regulate their phagocytosis by modulating trans presentation of 'self' (Fig.3A) – as is relevant to cancer therapy (Fig.1A). …

CRISPR/Cas9-mediated knockout (KO) of CD47 or SIRPα in B16 cells used a non-targeting guide-RNA for control, and all B16 cells were equally opsonized with anti-Tyrp1 (Fig.S1B,S4A) before adding to mouse bone-marrow derived macrophages (BMDMϕs). …

Reviewer 2: Two different metrics of phagocytosis are used, and it is not clear why. In Figures 1C, D "% Macrophage eating" is the metric, while in Figure 2 "# shRBCs per Macrophage" is the metric. It seems that the data in both figures could be reported with either metric. Is there a reason for the use of different metrics?

RESPONSE:
The different metrics give similar impressions as we had noted previously:

"The increased percentage of eating macrophages was also accompanied by more hRBCs eaten per macrophage (Fig.S2A, top)"

TEXT CHANGE:
"Phagocytosis was assayed (again per macrophage as in Fig.S2A, top), …."

Reviewer 2: In Figure 3, why does the anti-CD47 antibody not compete off SIRPα bound in trans? It seems that this competition should be the same as when pre-priming the macrophage with anti-CD47 antibody, in which case you see strong CD47 antibody binding. Reporting the anti-CD47 antibody binding affinity would also be helpful.

RESPONSE:
We agree with this possibility and explain this as motivation for knockdown:

"Anti-CD47 on a macrophage could conceivably dissociate and bind an RBC target and vice versa – although symmetric saturation of both cells will eliminate exchange and was seen to maximize eating (Fig.1D). To achieve a more stable asymmetric blockade effect, CD47 was suppressed in THP-1 macrophages using shRNA, without affecting SIRPα"

Reviewer 2: It would be helpful to report the absolute densities of CD47 and SIRPα molecules on the different cell types in Figure 1B. Also, labeling SIRPα and quantifying surface expression would be useful in validating the claims of this experiment in Figure 3A, and it could also validate that the CD47-SIRPα stoichiometry is in a reasonable and physiological range on the CHO cells.

RESPONSE:
Newly inserted Figure 1B-Table shows estimations calibrated against human RBC CD47 levels as cited:

Fig.1B … (Table) Molecular density estimates for CD47 and SIRPα on several hematopoietic cell types based on (Subramanian-et-al.,-2007).

Regarding the CHO cell experiments (now Fig.4A,B):

"Heterologous display of human CD47-GFP on Chinese hamster ovary (CHO) cells varies broadly, including physiological levels (Subramanian-et-al.,-2007), and co-expression (or not) of human-SIRPα (unlabeled but expressed similarly), allowed us to assess trans binding of anti-CD47 or soluble-SIRPα."

Reviewer 2: The authors use simulations to show that SIRPα-CD47 cis binding is possible in Figure 4 and S4B, but the likelihood of binding is cis vs trans is unclear. The authors' simulations would benefit from analysis of the energetic cost of bending versus binding, and an evaluation of how likely it is to find SIRPα molecules in this state. It seems at least possible that SIRPα on the macrophage would prefer to bind to CD47 on the RBC surface rather than CD47 on the macrophage if both were nearby (i.e. trans interactions could outcompete cis interactions).

RESPONSE:
Although more simulations seem beyond the scope of typical J Cell Science papers, they are certainly of interest and noted at the end of the Results & Discussion:

“Bending energy could destabilize binding energy, and a first step toward determining this might be to relate SIRPα-docking probabilities for CD47-derived ‘Self’-peptides to measured binding affinities (Rodriguez-et-al.,-2013).”

Reviewer 2: How does CD47 binding to SIRPα in cis drive phosphorylation of the ITIM domain?

RESPONSE:
This is a great question, but the pathway in trans is also poorly understood beyond binding.
We therefore wrote in the revised text at the end of the section “Knockdown of macrophage CD47 increases eating and decreases basal signaling”:

TEXT CHANGE:
“CD47 binding to SIRPα in trans certainly drives phosphorylation of the ITIM domain, and a similar mechanism presumably applies to CD47 binding to SIRPα in cis.”

Reviewer 2: Minor points:
It appears that the labels are switched for the two conditions in Figure 3B and C.

RESPONSE:
We apologize for the oversight and any confusion the mislabeling may have caused.
The plots are corrected as shown in Fig.4B.

Reviewer 2: Additionally, it is unclear how the data points for these plots were selected from flow cytometry data. Are they averages of populations?
These data points are an average of 10,000 events analyzed by flow cytometry. This clarification has also been added to the manuscript in the legend of Figure 4B:

TEXT CHANGE:
“Datapoints were generated by averaging 10,000 events from flow cytometry.”

Reviewer 2: Figure S3C – The y-axis is incorrect.

RESPONSE:
We again apologize for the mistake and have placed the correct label in this figure (now Fig. S3B).

Second decision letter

MS ID#: JOCES/2019/237800

MS TITLE: Macrophages eat more after disruption of *cis* interactions between CD47 and the checkpoint receptor SIRPα

AUTHORS: Dennis E Discher, Brandon H Hayes, Richard Tsai, Lawrence J Dooling, Siddhant Kadu, Justine Y Lee, Diego Pantano, Pia L. Rodriguez, Shyamsundar Subramanian, and Jae-Won Shin
ARTICLE TYPE: Short Report

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks. In addition, I would like to ask you to address the request from reviewer 2 in the discussion of your revised manuscript prior to final submission.

Reviewer 1

Advance summary and potential significance to field

The manuscript by Hayes et al. is a study of the interaction between CD47 and SIRPa on macrophages. Treatments that block the interaction between CD47 and SIRPa suppress tumor growth via different mechanisms including enhanced phagocytosis. In that case, the inhibitory receptor SIRPa does not engage with CD47 and is less active to prevent phagocytosis of the CD47-positive target cell. The role of the CD47 molecules expressed on macrophages has been overlooked so far and this is the focus of the present study. The authors convincingly demonstrated that CD47 inhibits phagocytosis via SIRPa in cis in model macrophage-like cells. The experiments are well controlled. The manuscript is of very good scientific standards and is well written. The results will be of interest in the field of immunomodulation but also from a cell biology point of view on receptor-receptor interactions.

Comments for the author

The authors have addressed my concerns and the manuscript has been much improved. As long as this reviewer is concerned, the manuscript can be accepted for publication.

Reviewer 2

Advance summary and potential significance to field

Interesting finding that cis interactions between CD47 and SIRPa on macrophages can affect phagocytosis.

Comments for the author

The authors have added useful new data and clarified many of my questions.

What remains unclear to me from the study is the potency of SIRPa signaling in cis versus trans. This is particularly relevant because the authors highlight in the introduction that accumulation of SIRPa to a phagocytic synapse is important in macrophage inhibition. The authors have added interesting new data in Figure 3 demonstrating that knocking out CD47 or SIRPa on a tumor target changes the ability of the other molecule to bind in trans, thus emphasizing the fact that modulating relative cis interactions can affect trans binding. However, this data does not address how a release of cis interactions on the macrophage changes phagocytosis, specifically why CD47 KD macrophages phagocytose more RBCs. There appears to be a lack of consistency between the phagocytosis results for macrophage KD of CD47 (which should increase SIRPa-CD47 trans interaction) and the tumor cell KO of SIRPa (which should also increase SIRPa-CD47 trans interaction). This suggests that a simple competition model does not fully explain the findings in the manuscript. It would be helpful for the authors to provide more discussion of this apparent conflict and any hypotheses that could explain the results.